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Assembly of the Precursor and Processed Light-harvesting Chlorophyll *a/b* Protein of *Lemna* into the Light-harvesting Complex II of Barley Etiochloroplasts

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Abstract. When the in vitro synthesized precursor of a light-harvesting chlorophyll *a/b* binding protein (LHCP) from *Lemna gibba* is imported into barley etiochloroplasts, it is processed to a single form. Both the processed form and the precursor are found in the thylakoid membranes, assembled into the light-harvesting complex of photosystem II. Neither form can be detected in the stromal fraction. The relative amounts of precursor and processed forms observed in the thylakoids are dependent on the developmental stage of the plastids used for uptake. The precursor as

well as the processed form can also be detected in thylakoids of greening maize plastids used in similar uptake experiments. This detection of a precursor in the thylakoids, which has not been previously reported, could be a result of using rapidly developing plastids and/or using an heterologous system. Our results demonstrate that the extent of processing of LHCP precursor is not a prerequisite for its inclusion in the complex. They are also consistent with the possibility that the processing step can occur after insertion of the protein into the thylakoid membrane.

THE light-harvesting complex of photosystem II (LHC II)¹ is the major protein component in the thylakoid membranes of higher plant chloroplasts (34). The most prominent polypeptides of this complex are the light-harvesting chlorophyll *a/b*-binding proteins (LHCPs). The LHCPs are encoded in the nucleus and are synthesized on cytoplasmic ribosomes as a soluble, higher molecular weight precursor form(s) (pLHCP) (31). The uptake and processing of pLHCP have been studied in vitro and shown to be posttranslational and ATP dependent (12, 31). In vitro translation products of poly(A) RNA can be taken up by isolated chloroplasts, and the processed form of LHCP has then been found associated with a chlorophyll-protein complex (4, 31). The first event in the uptake is presumably a recognition event between the precursor and a putative receptor on the outer envelope (10). After uptake, a 3–4-kD transit peptide is cleaved from the amino terminus (7, 27). The processing of the precursor of another nuclear coded chloroplast protein, the small subunit of ribulose biphosphate carboxylase, has been shown to occur in at least two steps in the stroma (29, 30). However, the location and mechanism of processing of other nuclear coded chloroplast proteins and the mechanisms by which the soluble precursors traverse the envelope membranes and by which

the proteins are targeted to their respective organellar compartments are not known.

Many events during the synthesis of the major light-harvesting complex (LHC) are affected by light. LHCP is absent from etioplasts of angiosperms and accumulates during the greening process (14–16, 33). The transcription of genes coding for LHCP can be regulated by phytochrome action (cf. reference 35). The accumulation of LHC II appears to be coordinated with that of chlorophyll (5, 14, 15, 33), whose synthesis is also light dependent. In addition, the structural development of the chloroplast is both triggered by and dependent on light. One might expect, therefore, that aspects of the import and processing of LHCP and of its assembly into LHC II might be related to plastid development.

We have been characterizing the LHCPs of *Lemna gibba* and the genes coding for them (36). *Lemna* thylakoids contain at least three immunologically related LHCPs with apparent sizes of 27–28 kD. They are encoded by a family of ~12 nuclear genes (36), two of which have been sequenced (19, 21). In the previous paper (21) we reported the in vitro expression of a single *Lemna* LHCP gene and the import of its protein product by *Lemna* etiochloroplasts.

In the work described here, the in vitro expressed *Lemna* pLHCP has been imported into etiochloroplasts isolated from greening barley seedlings to study the uptake, processing, and assembly of LHCP in a heterologous system. We selected barley because plastid development and chlorophyll biosyn-

¹ Abbreviations used in this paper: ALA, 5-aminolevulinic acid; LHC, light-harvesting complex; LHC II, LHC of photosystem II; LHCP, light-harvesting chlorophyll *a/b* protein; pLHCP, precursor of the light-harvesting chlorophyll *a/b*-protein; RB, resuspension buffer; SAM, S-adenosylmethionine.

thesis have been extensively studied in this species, and green-
ing barley leaves are a convenient source of etiochloroplasts
that can synthesize chlorophyll *in vitro* (17). We show that in
the heterologous system, both the precursor and processed
forms of the *Lemna* LHCP are present in LHC II of barley
thylakoid membranes. In addition, we find that the uptake
and processing of pLHCP are markedly dependent on the
stage of development of the isolated plastids.

Materials and Methods

Plant Material

Barley (*Hordeum vulgare* L. cv Poco; Arco Seed Company, El Centro, CA)
seedlings were grown in vermiculite at 25°C in complete darkness. Plastids were
isolated from etiolated 6-d-old seedlings which had been illuminated (30
 $\mu\text{Einsteins/m}^2$ per s^{-1}) for durations indicated in the figure legends. At this
stage of development, seedlings were 6–8 cm long, and the primary leaf had
emerged. 9-d-old maize (*Zea mays* L. cv. Golden Cross Bantam; W. Atlee
Burpee Company, Riverside, CA) seedlings, similarly grown, were used in some
experiments.

Isolation of Plastids

Barley leaves were harvested, cooled on ice, and cut into 2–3-cm sections before
grinding (seven 1-s bursts at 70% full power) in a razor blade blender (18) in a
buffer containing 0.6 M glycerol, 50 mM Tricine-KOH (pH 7.9), 1 mM MgCl_2 ,
and 1 mM dithiothreitol (10). The ratio of leaves to buffer was 1:5 (wt/vol).
The homogenate was filtered through a 30- μm nylon net (Nitex, Tetko Inc.,
Elmsford, NY). The plastids were pelleted by centrifugation in a Sorvall GSA
rotor by accelerating to 3,000 rpm and applying the brake when that speed was
reached. The pellet was gently dispersed in resuspension buffer (RB) which
contained 0.3 M glycerol, 100 mM Tricine-KOH, pH 7.9, 1 mM MgCl_2 . Equal
volumes of the resuspended plastids were transferred to 15-ml Corex tubes and
centrifuged in a Sorvall SS34 rotor by accelerating the rotor to 3,500 rpm and
then immediately braking. In a typical experiment plastids for each treatment
contained 40 μg chlorophyll and were obtained from ~35 g tissue. The pellets
were gently suspended in 100 μl RB and used for uptake.

Transcription and Translation

The *in vitro* expression of the AB30 gene to obtain [^{35}S]methionine-labeled
pLHCP was carried out as detailed by Kohorn et al. (21). No capping was
required for efficient translation of mRNA transcribed from the AB30 gene
cloned in the psp65 vector. The isolation of the S30 fraction from wheat germ
(28) had to be modified to include the later part of the first peak from the
Sephadex column in order to get translation of the AB30 mRNA. The nature
of the factor(s) thus added to the translation system was not determined.

In Vitro Uptake of the AB30 Precursor

The uptake mixture consisted of 100- μl plastid suspension, 125 μl translation
products, 20 μl of 1 M Tricine-KOH (pH 7.9), 5 μl of 50 mM ATP, and 10 μl
each of 6 M glycerol, 30 mM S-adenosylmethionine (SAM), 30 mM 5-
aminolevulinic acid (ALA), 250 mM methionine, and water. The plastids were
incubated for 60 min at a low light intensity (30 $\mu\text{Einsteins/m}^2$ per s^{-1}) and
24°C on a Tektator V shaker (American Hospital Supply Corporation, Miami,
FL) at 40 rpm. Higher light intensities were observed to reduce the amount of
labeled polypeptides found in thylakoids and could be expected to increase the
photobleaching of newly synthesized chlorophyll. To ensure that ATP, required
for import (12), was not limiting, it was supplied in the uptake mixture as were
ALA and SAM since they are known to be required for chlorophyll synthesis
in isolated plastids (17).

Isolation of Thylakoids After Uptake

Uptake was stopped by transferring the tubes to ice and adding 5 ml RB. The
plastids were pelleted by accelerating in an SS34 rotor to 3,500 rpm and
stopping it immediately when that speed was attained. They were then sus-
pended in 500 μl RB and incubated on ice for 30 min with 68 $\mu\text{g/ml}$ thermolysin
and 8 mM CaCl_2 . This procedure, aimed at digesting those precursor molecules
still bound to the plastid's outer envelope after import, was adopted from Cline
et al. (9), who showed that thermolysin digests only the polypeptides of the
outer envelope of chloroplasts and does not cross the outer envelope, unlike
trypsin and chymotrypsin which are more conventionally used for digesting

unincorporated precursor proteins (e.g., 13, 31). We observed that treatment
with trypsin and chymotrypsin markedly reduced the proportion of intact
plastids recovered as compared to treatment with thermolysin. At the end of
the protease treatment, the plastids were underlayered with 2 ml of 30% Percoll
containing 0.3% bovine serum albumin (fraction V), 0.3% Ficoll (400,000 D),
0.9% polyethylene glycol 8000, 0.3 M glycerol, 1 mM MgCl_2 , 5 mM tetrasodium
salt of EDTA, and 100 mM Tricine-KOH (pH 7.9). The tubes were centrifuged
in an SS34 rotor at 6000 rpm for 3 min. The etiochloroplasts obtained in the
pellet were found to be 70–75% intact by comparing the specific activity of
NADP glyceraldehyde-3-phosphate dehydrogenase (37), a stromal enzyme, in
the plastid preparation to that in the leaves. The pellet was suspended in 5 ml
RB containing 5 mM EDTA and pelleted again by accelerating in an SS34
rotor to 6,000 rpm. The plastids were then broken by vortexing in 1 ml of wash
buffer (5 mM EDTA and 50 mM Tricine-KOH, pH 7.9) and transferred to
microfuge tubes. The thylakoids were pelleted by spinning in an SS34 rotor at
15,000 rpm for 10 min. The stromal proteins were precipitated by adding 100%
trichloroacetic acid to the supernatant to a final concentration of 10%. They
were pelleted, washed once with acetone, solubilized, and then denatured. The
thylakoid membranes were resuspended in 1 ml wash buffer. 100 μl of the
suspension was removed for protein determination and the rest was centrifuged
again at 15,000 rpm. Typically, in each treatment, thylakoids containing ~70
 μg protein were recovered at the end of the experiment. The labeled polypeptides
found in the thylakoids constituted ~1% of the labeled polypeptides in which
the plastids had been incubated.

Treatment of Thylakoids with Trypsin or Alkali

The thylakoids were suspended in wash buffer and TPKC-treated trypsin was
added to the final concentration of 100 $\mu\text{g/ml}$. They were then incubated at
37°C for 10 min. The reaction was stopped by addition of soybean trypsin
inhibitor (Type I-S) to a concentration of 500 $\mu\text{g/ml}$. The conditions for trypsin
digestion were optimized using unlabeled thylakoids from plants illuminated
for 12 h and also using the previous work (32). The thylakoids were then
pelleted and denatured. Peripheral membrane proteins were washed from the
thylakoids by 0.1 M NaOH as described previously (31).

Protein Fractionation and Fluorography

The thylakoids were solubilized by suspending in 40 μl of a buffer containing
12.6 mM Tris, 96 mM glycine, and 20% glycerol, and then denatured by
incubating at 55°C for 1 h in the presence of 4% wt/vol SDS and 5% vol/vol
2-mercaptoethanol. Electrophoresis was carried out in a 10–16% linear gradient
polyacrylamide gel (22). The protein bands were stained with Coomassie Blue
R. Fluorography was performed according to the protocol of Laskey and Mills
(23).

Chlorophyll and Protein Determinations

Chlorophyll was extracted in 80% acetone containing 0.01 M NH_4OH . The
concentration of total chlorophyll was determined according to Arnon (3).
Protein was microassayed according to Bradford (6) using protein assay dye
reagent (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin (frac-
tion V) was used as a standard. Thylakoid samples were solubilized by incu-
bating them with 1% Triton X-100 for 15 min at 55°C prior to determining
their protein content.

Chemicals

Glycerol and inorganic chemicals were purchased from Mallinckrodt Inc., St.
Louis, MO; [^{35}S]methionine and SP6 polymerase were from New England
Nuclear, Boston, MA; and acrylamide was from Eastman Kodak Co., Roch-
ester, NY. Deriphat-160 was purchased from McKerson's Chemical Laborato-
ries, Minneapolis, MN. All other chemicals were obtained from Sigma Chemical
Co., St. Louis, MO.

Results

Association of pLHCP with Thylakoid Membranes

The pLHCP translation product synthesized from the AB30
RNA transcribed *in vitro* has the expected electrophoretic
mobility (corresponding to ~32 kD) (Fig. 1a). Fluorography
of polyacrylamide gels containing the separated proteins of
barley plastids that had been incubated with this *in vitro*
synthesized pLHCP showed two labeled polypeptides in the

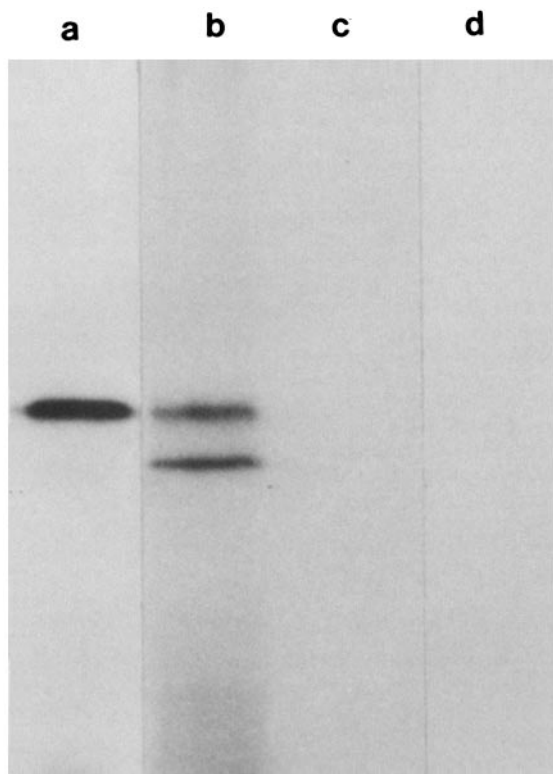


Figure 1. Uptake and processing of *Lemna gibba* pLHCP by barley etiochloroplasts obtained from plants that were illuminated for 8 h. Fluorograph shows (a) [³⁵S]methionine-labeled pLHCP obtained by in vitro translation of AB30 mRNA transcribed in vitro; (b) thylakoid proteins imported by barley etiochloroplasts after incubation with the translation product in lane a; (c) stromal proteins from the etiochloroplasts used to isolate thylakoids shown in lane b; (d) envelope fraction from similar plastids after uptake of pLHCP. Thylakoids, stroma, and envelopes were obtained from an equal number of plastids.

thylakoid fraction (Fig. 1 b). One of them corresponds to the pLHCP, while the other, having an apparent molecular weight of ~28,000, corresponds to the slowest migrating form of the mature LHCPs of *Lemna* and represents the processed form. Neither of these polypeptides could be detected in the stromal fraction (Fig. 1 c).

The detection in thylakoids of the precursor form of a polypeptide imported into chloroplasts is an unexpected observation. Therefore, it was important to test that its presence there was not an artifact of the methods used. One reason for finding pLHCP in the thylakoids could have been that the thylakoids were contaminated with chloroplast envelopes that had pLHCP sequestered in them. As thermolysin (added in the procedure) will only digest proteins in the outer envelope (9), any pLHCP located in the space between the outer and inner envelopes or associated with the inner envelope would be protected from the protease. To test whether this had occurred, we lysed intact plastids after uptake of pLHCP and thermolysin treatment by freezing and thawing, and isolated the envelopes by floating the broken plastids on 1.2 M sucrose (8). No precursor was detected in the envelope fraction (Fig. 1 c); it was found only in the thylakoids.

It could also be argued that the protease treatment of the plastids after pLHCP uptake might have been insufficient to digest all precursor not within the intact chloroplasts and,

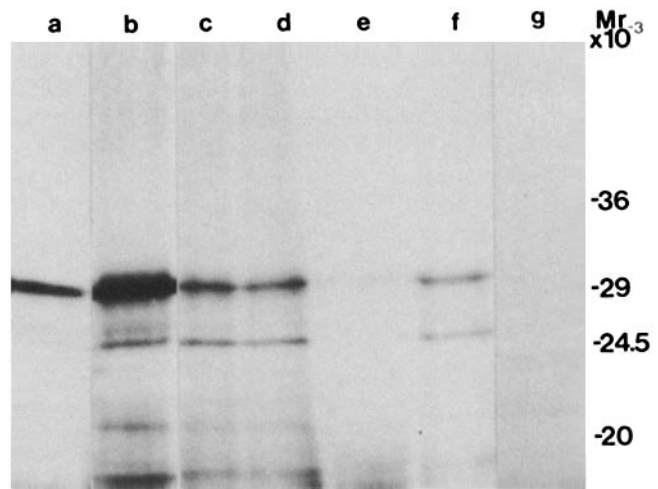


Figure 2. Presence of precursor is not due to insufficiency of protease treatment of plastids. Fluorograph shows (a) [³⁵S]methionine-labeled pLHCP obtained by in vitro translation of AB30 mRNA; (b–f) thylakoid proteins from plastids which were incubated with labeled pLHCP shown in lane a, and subsequently (b) kept intact and treated with no protease; (c) kept intact and treated with 68 μ g/ml thermolysin; (d) kept intact and treated with 180 μ g/ml thermolysin; (e) broken before treating with 68 μ g/ml thermolysin; (f) kept intact and treated with 455 μ g/ml trypsin and chymotrypsin; and (g) broken before treating with trypsin and chymotrypsin, 455 μ g/ml each. The positions of molecular weight markers are shown on the right. The plastids were isolated from plants that had been illuminated for 8 h.

further, that such precursor molecules were isolated together with the thylakoids. In the thylakoids obtained from plastids not treated with protease (Fig. 2 b), more than normal amounts of precursor could be detected (along with the processed LHCP). Treatment of plastids with two concentrations of thermolysin (68 μ g/ml and 180 μ g/ml) was used to digest any unincorporated precursor. Both concentrations reduced, but did not eliminate, the amount of precursor relative to that found in thylakoids of plastids not treated with protease. The higher concentration (Fig. 2, d), twice that normally used, does not give a significantly different result from the lower concentration (Fig. 2 c), demonstrating that the lower one suffices to eliminate any contaminating precursor. This conclusion is further substantiated by the fact that at the concentration routinely used (68 μ g/ml), both pLHCP and the processed form were eliminated from the membranes of plastids that had been deliberately broken before protease treatment (Fig. 2 e). We also treated plastids with other proteases, trypsin and chymotrypsin each at 455 μ g/ml. Exposing broken plastids to trypsin–chymotrypsin also resulted in digestion of both the precursor and the processed bands (Fig. 2 g), but when intact plastids were subjected to these proteases, the processed LHCP as well as the pLHCP could be detected in isolated thylakoids (Fig. 2 f). The reduced amounts of precursor and processed LHCPs remaining in these thylakoids could be because of breakage of plastids by these proteases (see above). These results indicate that the protease treatment is essential to prevent the inclusion of unincorporated pLHCP in the thylakoid sample, and that the treatment used was sufficient to eliminate such contamination. The thylakoids obtained from intact plastids showed the presence of some labeled polypeptides of low molecular weight. These are probably the result of the action of endogenous proteases.

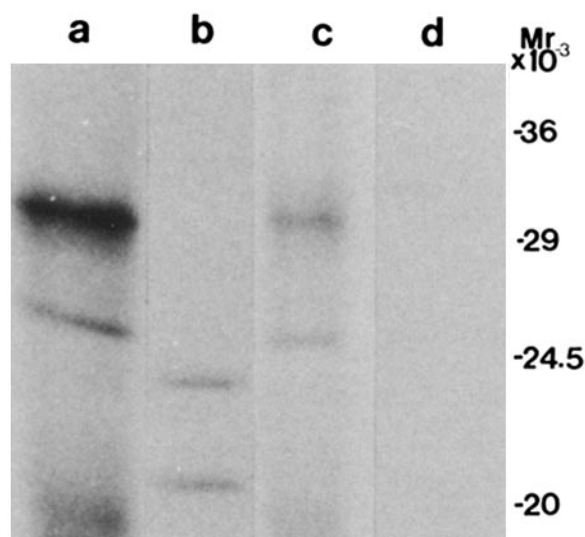


Figure 3. Evidence showing that pLHCP and LHCP behave as integral membrane proteins. Fluorograph includes (a) thylakoid proteins from plastids incubated with [35 S]methionine-labeled pLHCP; (b) proteins from thylakoids obtained from an equal number of plastids treated with 100 μ g/ml trypsin; (c) similar thylakoids washed with 0.1 M NaOH; and (d) the supernatant of NaOH wash. The lane M_r indicates position of molecular weight markers. The plastids were obtained from plants that had been illuminated for 8 h.

Other experimental data also support the observation that the precursor is inserted into the thylakoid membranes. For instance, when uptake was inhibited by incubating plastids in the dark on ice in the absence of ATP, pLHCP was not seen in the thylakoids (results not shown). Similarly, when uptake was stopped immediately after addition of translation product to the plastids, pLHCP (as well as the processed polypeptide) was absent from the thylakoids. Therefore, the precursor does not associate with the thylakoids in the absence of uptake.

Both the Precursor and Processed Forms of LHCP Are Integral Proteins of the Thylakoids

Integral thylakoid membrane proteins are resistant to proteolytic cleavage at those regions in the protein that are embedded in the lipid bilayer (1, 32). Also, they are not washed from the membrane by 0.1 N NaOH (31). Fig. 3 demonstrates that both forms of the LHCP incorporated into the thylakoids act like integral membrane proteins. The thylakoids of plastids that have imported labeled pLHCP contained precursor as well as processed forms of LHCP (Fig. 3a). When such thylakoids were treated with 100 μ g/ml trypsin at 37°C for 10 min, the apparent molecular weight of the native unlabeled LHCP was reduced by \sim 2 kD (data not shown), which is similar to what has been reported in pea (32) and *Lemna* (21), and the sizes of the labeled polypeptides were 1.5 and 4.5 kD smaller than LHCP (Fig. 3b). The precursor-product relationship between these trypsin digestion products (Fig. 3b) and pLHCP and LHCP (Fig. 3a) is not known. Note that the trypsin treatment here (Fig. 3) is milder than that used in the previous experiment (Fig. 2; cf. reference 31). When the thylakoids were incubated with 0.1 N NaOH after uptake of pLHCP and the membranes were re-isolated, both of the labeled polypeptides were recovered in the membrane fraction and neither was eluted by the NaOH (Fig. 3, c and d). The peripheral thylakoid proteins, e.g., polypeptides of coupling

factor I, were found in the alkali supernatant by analytical PAGE (data not shown). These experiments show that the labeled pLHCP and the LHCP are inserted in the lipid bilayer, but our experiments using trypsin suggest that the precursor is in a different conformation than the mature LHCP.

Both Precursor and Processed LHCP Are Found in LHC II

To test whether the incorporated LHCP polypeptides are assembled into the major light-harvesting complex (LHC II), the thylakoid chlorophyll protein complexes were prepared using a modification (Peter, G. and J. P. Thornber, manuscript in preparation) of the PAGE system described by Markwell et al. (24). For the data shown in Fig. 4, thylakoids obtained from intact plastids after the uptake of labeled pLHCP were solubilized in octyl glucoside/nonyl glucoside/SDS/chlorophyll (15:15:1:1; wt/wt) and run for 30 min through a 6.5% polyacrylamide gel. An important advantage of this system is that essentially all of the chlorophyll and carotenoid molecules in the surfactant extract of thylakoids from processed tissue are found complexed with proteins after electrophoresis. This electrophoretic system resolved two green LHC II bands along with other photosystem I and photosystem II complexes and a small amount of free chlorophyll when thylakoids isolated from plastids of plants illuminated for 12 h were used (Fig. 4A). A strip of gel containing this separation of green complexes was run on a fully denaturing gel to provide a two-dimensional analysis (Fig. 4A). The fluorograph shows that the LHC II bands contain the precursor form as well as the processed polypeptide (Fig. 4B). The presence of pLHCP in the complex shows that, at least in this heterologous system, processing is not a prerequisite for the LHCP to be included in the chlorophyll-protein complex.

Uptake and Processing Is Dependent on the Stage of Plastid Development

During the course of our experiments, we noticed significant variation in the proportion of precursor to processed LHCP incorporated into the thylakoids (compare Figs. 1–4). The stage of greening of the plants from which the plastids were isolated was the major factor that differed in these experiments. Therefore, we tested whether the stage of plastid development has any effect on the uptake and processing of pLHCP. The results of such an experiment are shown in Fig. 5. The increase in chlorophyll content of the plastids during this time of greening is over 100-fold (Fig. 5B). On the other hand, changes in protein content of plastids during greening are relatively small (data not shown; see references 16 and 20). Thus, to compare amounts of pLHCP and LHCP incorporated by plastids at different stages of development, we loaded equal amounts of protein, rather than chlorophyll, so that the number of plastids represented in each lane would be similar.

Plastids were isolated from one batch of barley seedlings that had been illuminated for different lengths of time. Marked differences occurred in the relative amounts of precursor and processed LHCP present in thylakoids after different times of greening (Fig. 5A). Precursor, but hardly any processed LHCP, was detected in thylakoids isolated from plastids obtained from etiolated leaves (Fig. 5A, lane 0) and

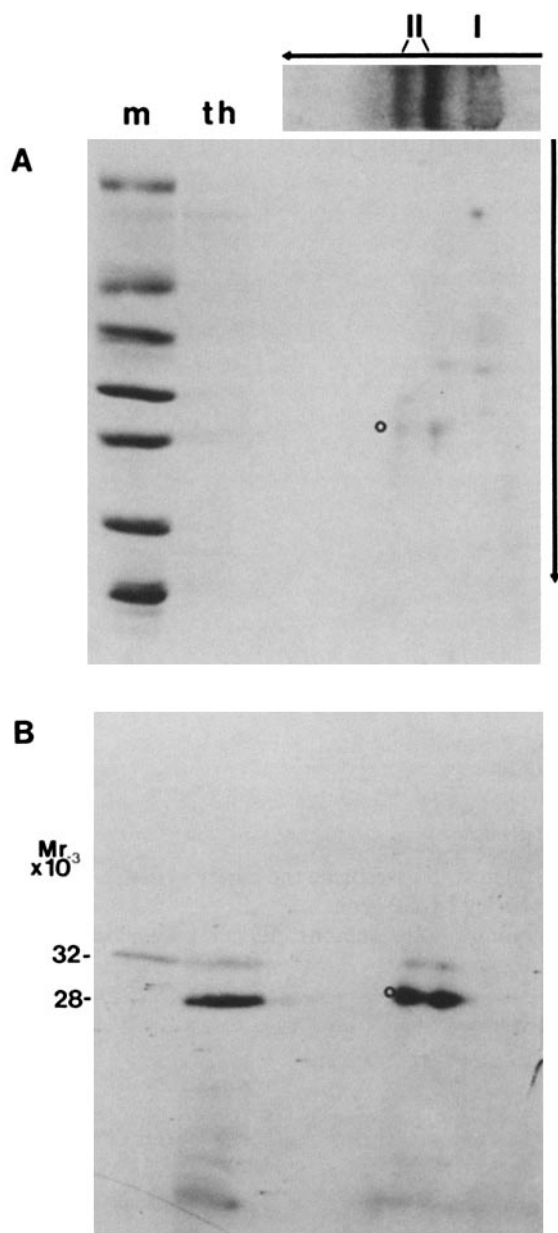


Figure 4. Incorporation of *Lemna* pLHCP into LHC II of barley etiochloroplasts which were isolated from plants illuminated for 12 h. The horizontal arrow represents the direction of migration during partially denaturing gel electrophoresis that fractionates thylakoids into one photosystem I (I) and two LHC II (II) bands along with other minor bands. A strip of this gel was placed on top of a fully denaturing gel and subjected to electrophoresis in the direction of vertical arrow. This second gel was stained with Coomassie Blue (A), and then fluorographed (B). Also shown in the second dimension are (m) molecular weight markers ($M_r \times 10^{-3}$) of 66, 45, 36, 29, 20, 14 including wheat germ translation products of AB30 RNA, and (th) a thylakoid preparation from etiochloroplasts incubated with pLHCP. The position of LHCP is indicated by o.

from those greened for 2 h. Labeled, processed LHCP was easily detected after 5 h of exposure of the etiolated tissue to light and the amount of it detected in the membrane increased up to 12 h, decreasing again thereafter (Fig. 5A). Uptake of pLHCP was observed throughout the greening process, i.e., until 18 h. It was prominent in etiochloroplasts, increased in amount up to 8 h of greening, and reduced later. No label was detected

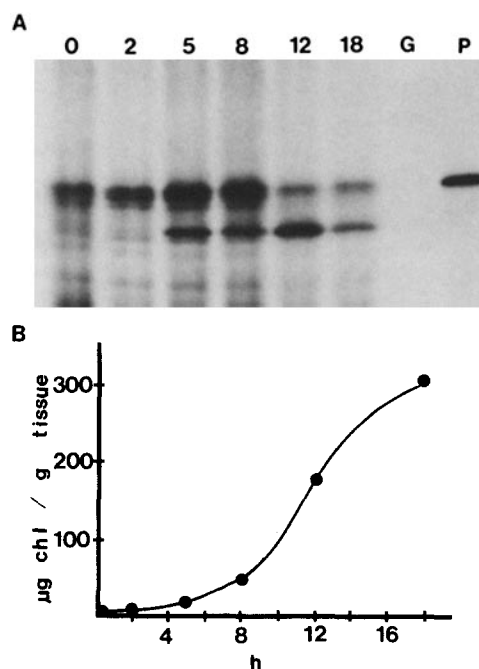


Figure 5. The amounts of pLHCP and LHCP in the thylakoids depend on the developmental stage of plastids used for import of pLHCP. Fluorograph (A) shows pLHCP and LHCP in the thylakoids obtained from plastids which were incubated with pLHCP, and which were isolated from plants illuminated for 0, 2, 5, 8, 12, 18 h and also from plants grown in continuous light (lane G). Lane P shows wheat germ translation products of AB30 mRNA. B shows accumulation of chlorophyll during the time of greening.

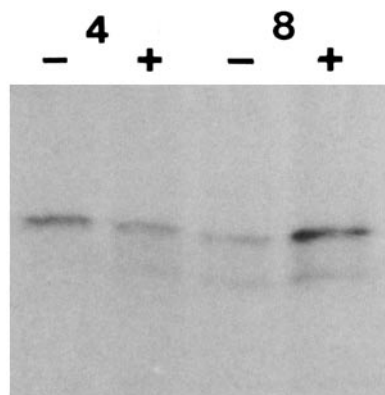


Figure 6. Effect of the developmental stage of plastids and of the presence of ALA and SAM during uptake of pLHCP by maize plastids. Fluorograph shows pLHCP and LHCP in the thylakoids obtained from plastids isolated from plants illuminated for 4 and 8 h. The plastids were incubated with pLHCP in the presence (+) or absence (-) of ALA and SAM (1 mM each).

in plastids from leaves of light-grown seedlings of the same age as the greening leaves (Fig. 5A, lane G). Additional labeled bands of a smaller apparent size were evident mainly during the early stages of greening (Fig. 5A). These are presumably degradation products of either the precursor or the processed form. However, it is possible that one of these bands of ~30 kD is an intermediate in the processing of pLHCP to the processed form (cf. reference 30).

The uptake and processing of *Lemna* pLHCP by maize etiochloroplasts was also found to be dependent on the stage of plastid development (Fig. 6). The plastids obtained from

the etiolated plants illuminated for 4 h contained less processed LHCP in the thylakoids than the plastids from plants illuminated for 8 h. In addition we observed that the presence of ALA and SAM during uptake (Fig. 6, +) increased the amount of LHCP recovered in the maize thylakoids. However, the effect of ALA and SAM was not substantial in barley (data not shown).

Discussion

Both the precursor and processed forms of *Lemna* LHCP have been detected as integral membrane proteins of the major LHC of thylakoid membranes after import of *Lemna* pLHCP by isolated, intact barley plastids. Although the uptake of pLHCP by chloroplasts and incorporation of LHCP into a chlorophyll-protein complex has been reported by other workers (4, 10, 31), there is no report of pLHCP being found inside the chloroplasts either in the stroma or in thylakoids. However, data presented by Cline et al. (10) who studied uptake of translation products of poly(A) RNA hybrid-selected with a cloned pea LHCP sequence, do show the presence of small amounts of radioactivity in the region where pLHCP would run. The results described in our paper clearly show that imported pLHCP is located in the thylakoids. Alternative interpretations of such an observation, namely that its presence in the thylakoids is due to insufficient protease treatment of intact plastids or to contamination of thylakoids by chloroplast envelope membranes containing pLHCP, have been eliminated. Furthermore, pLHCP is not only inserted into the thylakoids but it is also incorporated into LHC II.

The manner in which pLHCP, a water-soluble protein, is folded in the membrane is not known. Trypsin treatment of barley membranes having an LHC II containing imported *Lemna* pLHCP and also its processed form indicates that these polypeptides have slightly different regions accessible to the protease than native barley LHCP. The site and nature of processing of pLHCP is also not defined, although pLHCP's presence in the thylakoids suggests that processing might take place on these membranes. LHCP is not a water-soluble protein and so it is reasonable to expect it to be processed at or near its final destination. There is evidence that another thylakoid membrane protein, albeit one synthesized within the chloroplast, is processed after its insertion into the membrane (11). However, direct evidence to support the view that pLHCP is processed on the thylakoid membrane is lacking. Nevertheless, the presence of pLHCP in LHC II indicates that processing is not required for its inclusion in the complex.

Chloroplast biogenesis involves intricate biochemical and morphological changes triggered by light (35). As etioplasts are exposed to light, not only is the synthesis of pLHCP and chlorophyll triggered but so also is the machinery for the uptake and processing of pLHCP. Immature plastids from lettuce have been found to be more efficient than chloroplasts from 10-d-old light-grown pea seedlings in taking up labeled pLHCP (31). In the present work, we report differences in the relative amounts of imported pLHCP and processed LHCP found in the thylakoids at various stages of greening (Figs. 5 and 6). These could be explained in several ways: (a) The synthesis or activity of the processing enzyme for pLHCP could be under the control of light. Thus, the uptake mechanism might develop prior to the processing activity which

could result in a transient accumulation of the precursor at early stages; (b) the stability of the processed form could be dependent on the developmental stage of the plastid. In this case one would imagine that during early stages of greening, processing is normal, but the processed polypeptide is immediately degraded because of a lack of chlorophyll (cf. references 2, 4, 5; however, see reference 25). In this context, note that significant amounts of chlorophyll (and the processed form) can be observed only after 5 h of greening (Fig. 5); (c) the composition of developing membrane might affect the conformation of pLHCP and its accessibility to the processing enzyme (if indeed the protein is processed at this location). Our preliminary observations (not shown) indicate that pLHCP can be more readily digested by trypsin or extracted by NaOH from thylakoids isolated from plastids of plants greened for 5 h than from those of plants greened for 12 h (Fig. 3 shows tissue greened for 12 h).

The precursor is observed in the thylakoids when either barley or maize plastids, but not when *Lemna* etiochloroplasts (21), import *Lemna* pLHCP. It is conceivable that the processing enzyme is more slowly induced during greening in barley and maize than in *Lemna*, or perhaps the specificity of the processing enzyme differs between species (cf. reference 26). It should also be noted that the *Lemna* pLHCP is processed to a single polypeptide in both barley and maize, while in *Lemna* it is processed/modified to three polypeptides that differ in apparent size (21). This observation suggests that there may be a more stringent species specificity for modifications of processed LHCP than for the initial processing step. It will be of interest to investigate the barley system with the product of a barley LHCP gene.

In summary, our results demonstrate that precursor LHCP can be inserted into the thylakoid membranes as a member of a stable LHC. We cannot tell if either the precursor or processed form itself binds pigment molecules; similarly, we cannot tell if the newly formed complex functions in energy transfer. The accumulation of both precursor and processed LHCP and the degree to which the precursor is processed are related to the extent of greening undergone by the barley and maize plastids. Since plastid chlorophyll content of plastids also increases with time, we cannot overlook the intriguing possibility that the increase in chlorophyll biosynthesis and the increase in processing activity are related.

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